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RESEARCH ARTICLE

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A systematic study of some lichenized fungi from Pakistan using nrITS marker

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Abstract

In a detailed investigation of lichen diversity in different regions of Pakistan, utilizing molecular and morphological techniques, we identified and reported three species – *Dimelaena altissima*, *Lecanora chlarotera*, and *Usnea lapponica* – as new records for Pakistan. Furthermore, nrITS sequences for *Evernia prunastri*, *Lecanora horiza*, *L. hybocarpa*, *Peltigera polydactylon*, and *P. praetextata* were generated for the first time from Pakistan. Detailed morpho-anatomical descriptions, habitat information, distribution data, and results of biochemical analyses are also provided for a better understanding of the lichen diversity and ecology in this region.

Keywords

Crustose, foliose, Gilgit-Baltistan, Khyber Pakhtunkhwa, morphology, taxonomy

Introduction

Pakistan's diverse landscapes, ranging from extensive forests in the northern mountains and mineral-rich terrain in the west to fertile agricultural regions in the east, create a varied geography and climate that support extensive biodiversity (Baig and Al-Subaiee 2009; Alam et al. 2011; Malik et al. 2012). Geographically, the country lies in South Asia, between 23.5°N and 40°N latitudes and 60°E and 80°E longitudes, with a primarily tropical continental climate that exhibits distinct seasonal variations (Farooqi et al. 2005; Akhtar et al. 2022).

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Pakistan likely hosts a significant diversity of lichens, yet much of it remains unexplored due to limited surveys conducted across various regions (Fayyaz et al. 2022). The taxonomic exploration of lichens in the region was initiated by Sultan Ahmad in 1965 and was followed by Hawksworth and Mehmood (1971), recording 10 species from coniferous forests and Iqbal et al. (1978), adding 17 new species through their work on macrolichens. The first comprehensive checklist of lichens of Pakistan, published by Aptroot and Iqbal (2012), includes 368 species belonging to 114 genera identified using morpho-anatomical characteristics. Since the initiation of ITS-based phylogenetic analysis of lichens in Pakistan, numerous new species have been identified across various regions and as of now, more than 440 lichen species have been documented from the country (Habib et al. 2017; Khan et al. 2018; Fatima et al. 2021; Fayyaz et al. 2022; Afshan et al. 2023; Din et al. 2024; Usman et al. 2024; Zulfiqar et al. 2024). The present study is a contribution to the lichenological exploration of Pakistan mainly based on collections from Gilgit-Baltistan and Khyber Pakhtunkhwa, Pakistan. This study not only expands the knowledge of lichen flora within the country but also provides a more comprehensive understanding of their distribution patterns and ecology.

Materials and methods

Morphological and chemical studies

Collections were made during a lichen survey of different areas of Khyber Pakhtunkhwa and Gilgit Baltistan, Pakistan, in the years 2020 and 2023, respectively. Morphological characters were observed under a stereomicroscope (Meiji Techno, EMZ-5TR, Japan). The chemistry of the thallus was determined by using standard K (5% KOH), C (commercial bleach), and KC (K followed by commercial bleach) and secondary metabolites were detected by thin-layer chromatography in solvent C as described by Orange et al. (2001). Measurements of anatomical features were made from freehand sections of apothecia mounted in water on a glass slide. The sections were observed using a compound microscope (MX4300H, Meiji Techno Co., Ltd., Japan).

DNA extraction and PCR amplification

Genomic DNA was extracted directly from a portion of thallus with apothecia from each specimen using a modified 2% CTAB method (Gardes and Bruns 1993). Extracted DNA was used for PCR amplification of the ITS nrDNA marker using primer pair, i.e., ITS1F forward primer (5′ CTTGGTCATTTAGAGGAAGTAA 3′) (Gardes and Bruns 1993) and ITS4 reverse primer (5′ TCCTCCGCTTATTGATATGC 3′) (White et al. 1990). PCR products were visualized with the help of a 1% agarose gel using ethidium bromide through a Gel documentation system (Sambrook and Russell 2006). PCR products were cleaned and sent for sequencing to Tsingke, China.

Phylogenetic analysis

The ITS region was amplified and sequenced for all specimens. Forward and reverse sequences were reassembled using the BioEdit Sequence Alignment Editor (Hall 2011). Initial identity verification was performed via BLAST searches at NCBI (https://www.ncbi.nlm.nih.gov/guide). ITS datasets were constructed by retrieving relevant sequences from the GenBank database. Alignment and manual editing were completed using BioEdit (Hall 2005). Phylogenetic analyses were conducted on the CIPRES Science Gateway (https://www.phylo.org/) using RAxML-HPC2 on XSEDE (version 8.2.10) with the GTR+GAMMA nucleotide substitution model. One thousand rapid bootstrap replicates were run to infer the evolutionary history of each species. The resulting Maximum Likelihood phylogenetic trees were visualized using FigTree v. 1.4.2 (Rambaut 2014) and subsequently edited in Adobe Illustrator CC 2021. Appropriate species were selected as outgroups to root each phylogenetic tree.

Results

Taxonomy

***Dimelaena altissima* (H. Magn.) M. Ai & Xin Y. Wang, Phytotaxa 574 (4): 259 (2022)**
Fig. 1

Description. Thallus crustose, areolate-placodioid, up to 5 cm across, well-defined, lobate. Areoles: strongly convex, rounded to irregular, 0.5–1 mm in diameter, radiating towards margins, up to 1.5 mm long, 0.5 mm wide. Color: yellowish green to yellowish white when dry, no change when wet. Upper cortex: 2-layered, outer greyish, 10–20 µm thick, inner hyaline, 20–30 µm thick, cells rounded, 5–8 µm in diameter. Algal layer: continuous, even, 60–80 µm thick, photobiont chlorococcoid, cells globose, 9–13 µm in diameter. Medulla: 90–120 µm thick, hyphae hyaline, 3–4 µm wide. Apothecia: frequent, lecanorine, 1–3 per areole. Disc: round, black, pruinose, plane to concave, 0.2–0.7 mm in diameter. Margins: thick, persistent, concolorous to thallus. Epihymenium: brown, 10–15 µm tall. Hymenium: hyaline, 40–50 µm tall. Hypothecium: hyaline, 60–70 µm tall. Paraphyses: septate, branched, capitate, 1–2 µm wide apically. Asci: hyaline, clavate, 40–50 × 15–20 µm. Ascospores: brown, 1-septate, 9–12 × 5–8 µm.

Chemical study. K+ve (yellow), C-ve, KC+ve (yellow). Usnic acid and norstictic acid detected through TLC.

Phylogenetic analysis. For phylogenetic analysis, sequences of the genus *Dimelaena* were retrieved from GenBank based on their similarity to our sequences and those used in a study on the phylogeny of *Dimelaena* by Ai et al. (2022). Due to the limited availability of *Dimelaena* sequences, some sequences of the closest genus *Buellia* were also included in constructing the phylogenetic tree. The final aligned file was comprised of 525 nucleotides, among which 345 were conserved, 180 were variable, 161

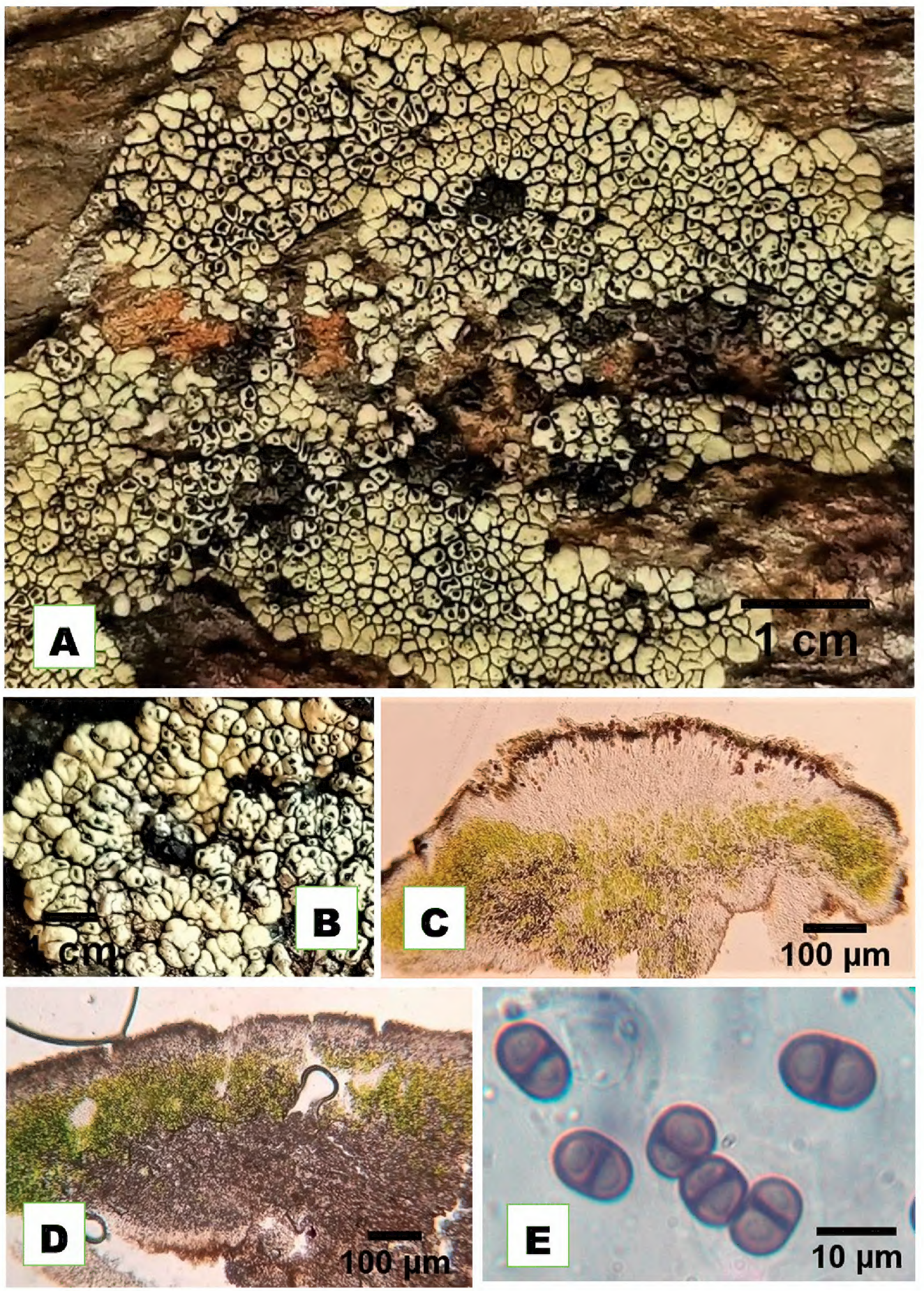

Figure 1. *Dimelaena altissima* **A** crustose-placodioid thallus **B** apothecia **C** cross-section of apothecium **D** cross-section of thallus **E** ascospores.

were parsimony-informative, and 19 were singleton variants. *Physcia aipolia* (Ehrh. ex Humb.) Fürnr. (MK811989 and MK812087) was chosen as an outgroup.

In our analysis, the newly generated sequences of *Dimelaena altissima* from Pakistan (KH-608, KH-609, and BST-03) grouped within a single clade alongside other se-

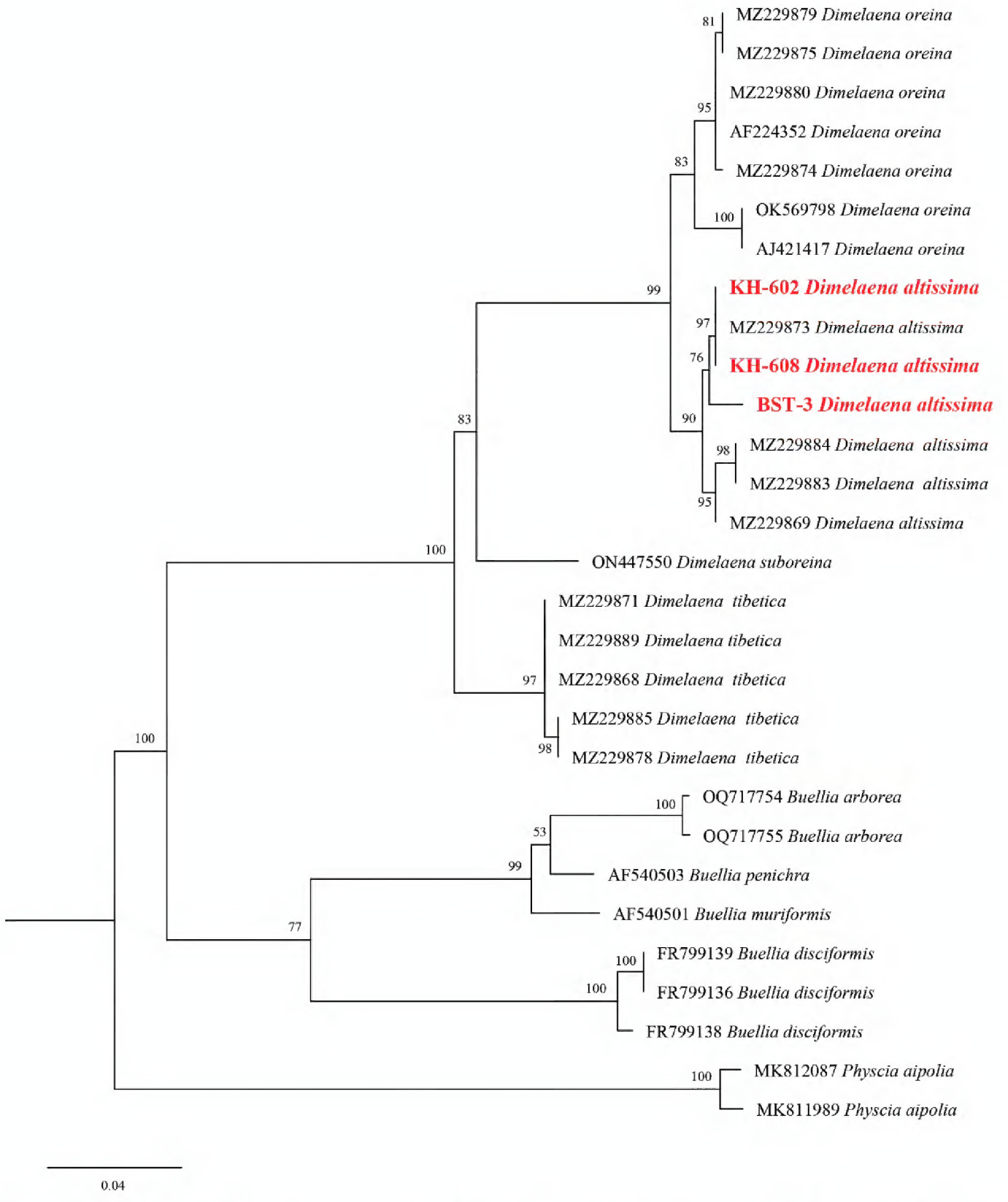

Figure 2. Molecular phylogenetic analysis of *Dimelaena* spp. by maximum likelihood (ML) method based on nrITS sequences. Bootstrap values >50% based on 1000 replicates for ML are shown above the branches and novel sequences generated during in this study are in bold.

quences of the same species (MZ229869, MZ229873, MZ229883, and MZ229884), receiving strong support (90% ML), thus confirming their identification as *D. altissima* (Fig. 2). Additionally, *D. altissima* shows a sister relationship with *D. oreina*, which also forms a distinct clade with high bootstrap support (83% ML).

Notes. *Dimelaena altissima* is distinguished by its yellowish-green, epruinose crustose thallus with flat marginal lobes, along with irregularly rounded, innate apothecia that have indistinct margins (Ai et al. 2022). The morphological comparison confirms

the identity of the Pakistani taxon as *D. altissima* as it shares same morpho-anatomical characteristics with *D. altissima* described from China, such as a crustose thallus with radiate-plicate margins and lecanorine immersed apothecia (Ai et al. 2022). It is morphologically and phylogenetically closely related to *D. oreina* but differs from it by lacking well-defined apothecial margins and convex marginal lobes (Ai et al. 2022). It is the first report of *D. altissima* from Pakistan.

Habitat and ecology. The collections (KH-602 & KH-608) were found in dry temperate climate, in an open situation exposed to sun and rain, at an altitude of 841 m a.s.l., temperature typically varies from -8 °C to 28 °C, with annual rainfall varying between 700–800 mm. Whereas the other collection (BST-03) was found at an elevation above 4,173 m, on rock, in a temperate climate with minimum and maximum temperatures of -8 °C and 35 °C, respectively. The region is characterized by hilly topography, with annual rainfall varying between 90 and 150 mm and heavy snowfall during the winter months.

Distribution. This species was previously documented from China (Ai et al. 2022) and is now recorded from Pakistan for the first time.

Materials examined. Pakistan • Khyber Pakhtunkhwa Province, Kohistan, Dassu; 35°35.0'N, 73°37.0'E; alt. 841 m a.s.l.; 11 Sep. 2020; K. Habib & A. N. Khalid leg.; on rock; KH-602 & KH-608, ITS GenBank accession numbers PQ113699 and PQ113700. Pakistan • Gilgit-Baltistan, District Diamer, Babusar Top; 35°08.8'N, 74°02.9'E; alt. 4,173 m a.s.l.; 28 Aug. 2023; U. F. Ahmad, M. Usman & A. N. Khalid leg.; on rocks; in moist temperate forest; BST-03, ITS GenBank accession number PQ113698.

***Evernia prunastri* (L.) Ach., Lich. Univ.: 442, tab. 10, fig. 1 (1810)**
Fig. 3

Description. Thallus foliose but appearing fruticose, dorsiventral, tufted, caespitose to occasionally pendent, cortex cracked at some places, palmately to dichotomously branched, up to 5.5 cm across. Branches: numerous, dorsiventral, elongated, 0.3–4 mm wide, plane, strap-shaped, pitted, twisted, tangled, blunt or pointed. Axils: right angles to V-shaped. Color: greyish-green to greenish-white when dry, pale olive green to yellow-green when wet. Soredia: marginal to rarely laminal, white, often on ridges, granular. Upper surface: matt, dull, pitted, strongly rugose, rough, ridges discontinuous**.** Lower surface: white to pale yellow, channelled, less pitted and wrinkled than upper surface. Cortex: two-layered, upper layer brown, 9–14 µm high, lower layer hyaline, 7–12 µm high, paraplectenchymatous, cell 7–12 µm in diameter. Medulla: prosoplectenchymatous, hyphae loosely arranged, white, 2–4.5 µm wide. Algal layer: discontinuous, uneven, forms small groups, algal cells present below cortex, 5–10 µm in diameter. Apothecia: not observed.

Chemical study. K +ve (yellow), C -ve, KC +ve (yellow). Usnic acid, atranorin and evernic acid detected through TLC.

Phylogenetic analysis. For phylogenetic analysis, sequences of the genus *Evernia* were retrieved from GenBank based on similarity and those used in a study by Piercey-Normore (2006). A total of 22 ITS nrDNA sequences were analyzed during this study. The final aligned file was comprised of 532 nucleotides, among which 359 were con-

Figure 3. *Evernia prunastri* **A** thallus **B** upper surface of the thallus **C** lower surface **D** granular soredia **E** cross-section of thallus **F** photobiont.

served, 158 were variable, 150 were parsimony-informative, and 8 were singleton variants. *Umbilicaria kappenii* Sancho, B. Schroet. & Vallad. (AY603130 and AY603131) was chosen as an outgroup.

In our analysis, *Evernia prunastri* forms a distinct clade and the ITS sequences of *E. prunastri* (KUM-04 and KUM-09) from Pakistan are identical to those reported from Norway (MK811838, MK811993, MK812247, and MK812058), confirming their identification as the same species (Fig. 4). Additionally, *E. divaricata* was distinctly separated from *E. mesomorpha* and *E. esorediosa* in the phylogenetic tree, yet all three species formed a clade with a high bootstrap support (92% ML). This study represents the first molecular confirmation of *E. prunastri* from Pakistan.

Notes. *Evernia prunastri*, commonly known as oakmoss, is a fruticose lichen found predominantly on exposed tree trunks, characterized by flat, isotomic-dichotomous

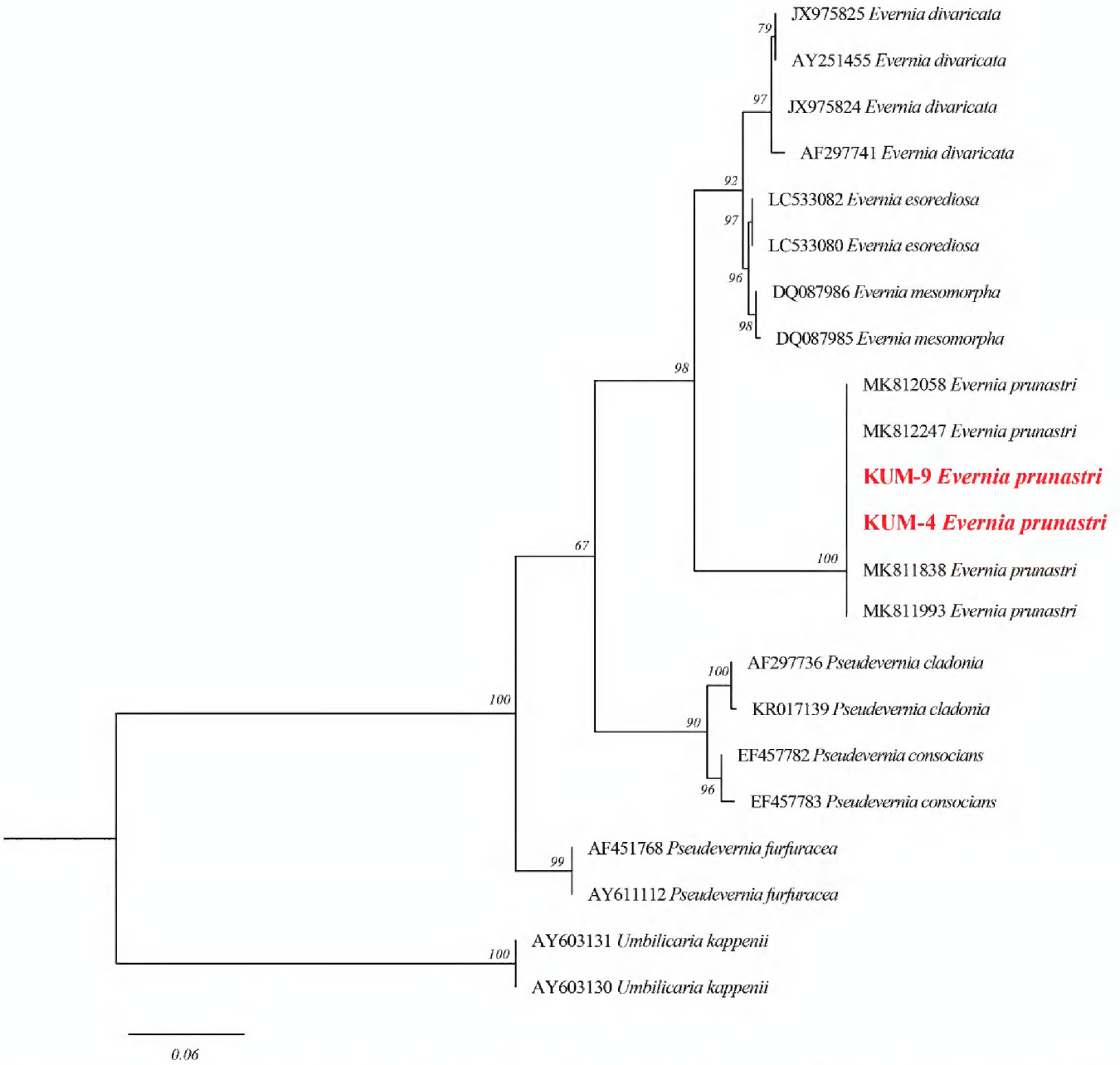

Figure 4. Molecular phylogenetic analysis of *Evernia* spp. by maximum likelihood (ML) method based on nrITS sequences. Bootstrap values >50% based on 1000 replicates for ML are shown above the branches and novel sequences generated during this study are in bold.

branches with frequent anisotomous bifurcations and occasional adventive branches or "lobuli" on the branch surface (Beltman 1978; Stone and McCune 1990). The morpho-anatomical comparison of Pakistani collections confirms their identity as *E. prunastri*, as the characteristics of Pakistani samples match with the already published description of *E. prunastri* from Britain and Ireland (Cannon et al. 2023).

Habitat and ecology. The samples were found at an elevation above 2000 m, growing on bark in the cold temperate mountainous regions. The coldest month is December, while June is the hottest month of the year, with average monthly temperatures of 3 °C and 25 °C, respectively, and rainfall varying between 600 and 1200 mm. The dominant vegetation includes Indian horse-chestnut (*Aesculus indica* (Wall. ex Cambess.) Hook.), deodar (*Cedrus deodara* (Roxb. ex D.Don) G.Don) and West-Himalayan fir (*Abies pindrow* (Royle ex D.Don) Royle).

Distribution. This species is widely distributed in Northern hemisphere including Asia, Europe and North America (Studzińska-Sroka et al. 2023; Staples et al. 2020).

Materials examined. Pakistan • Khyber Pakhtunkhwa Province, District Upper Dir, Kumrat Valley, 35°23.2'N, 74°35.0'E; alt. 2,500 m a.s.l.; 23 Sep. 2023; M. Usman & A. N. Khalid leg.; on bark; KUM-4, ITS GenBank accession number PQ113696 • same collection data as for preceding, 35°33.9'N, 72°11.4'E; alt. 2,400 m a.s.l.; 23 Sep. 2023; M. Usman & A. N. Khalid leg.; on bark; KUM-9, ITS GenBank accession number PQ113697.

***Lecanora chlarotera* Nyl., Flora, Regensburg 55(35): 550 (1872)**
Fig. 5

Description. Thallus crustose, verrucose-areolate to rimose-areolate, up to 1 cm across, indeterminate, continuous, irregular to orbicular, esorediate. Areoles: verruculose, smooth to rough, warty. Upper surface: greenish-white to yellowish-grey, smooth to rough. Prothallus: absent. Algal layer: 100–130 µm thick, even, continuous, cells chlorococcoid, spherical to sub-spherical, 6–15 µm in diameter. Apothecia: lecanorine, frequent, scattered to aggregated, sessile, epruinose. Disc: orange to reddish-brown, smooth, concave to slightly convex, rounded, 0.3–0.8 mm in diameter, epruinose to slightly pruinose. Margin: distinct, entire, smooth, thick, concolorous to thallus, persistent, continuous. Thalline exciple: well-developed. Paraphyses: hyaline, unbranched, 1.5–2 µm wide, simple, apically swollen, apex 3–4 µm wide. Epihymenium: reddish-brown to brownish-orange, 6–18 µm tall. Hymenium: hyaline, 70–100 µm tall. Hypothecium: hyaline, 25–42 µm tall. Asci: *Lecanora*-type, hyaline, thin-walled, sub-cylindrical to clavate, 8-spored, 60–78 × 10–15 µm. Ascospores: ovoid to broadly ellipsoidal, hyaline, simple, aseptate, 12–15 × 6–9 µm.

Chemical study. K +ve (yellow), C -ve, KC -ve. Atranorin was detected through TLC.

Phylogenetic analysis. For phylogenetic analysis, sequences of the genus *Lecanora* were retrieved from GenBank based on their similarity to our sequences. A total of 48 ITS nrDNA sequences were analyzed during this study, including four newly generated sequences. The final aligned file was comprised of 559 nucleotides, among which 285 were conserved, 268 were variable, 251 were parsimony-informative, and 17 were singleton variants. *Lecanora saxigena* Lendemer & R.C.Harris (KP224466 and KP224467) was chosen as an outgroup.

In our analysis, distinct clades were formed with high bootstrap values (Fig. 6). The newly generated sequences of *Lecanora chlarotera* (KUM-18 and SN-07) and *Lecanora hybocarpa* (FM-22) form a well-supported clade (99% ML) alongside *Lecanora paramerae* I.Martínez, Aragón & Lumbsch. The Pakistani sequences of *L. chlarotera* (KUM-18 and SN-07) cluster with sequences of the same taxon from the United Kingdom (FR799201, FR799204, FR799203) and Sweden (MW375013, MW375016, MW375017, MW375018, and MW374998), indicating they represent the same species. Additionally, the sequence of *L. hybocarpa* (FM-22) groups with a sequence of the same taxon from Spain (EF105412) with strong bootstrap support (89% ML). The sequence of the Pakistani collection (K-165) forms a clade with *Lecanora horiza* sequences reported from North Macedonia (OQ717441), Switzerland (KX132998), and the USA (MG554692), indicating they represent the same species. *L. horiza* is closely related to

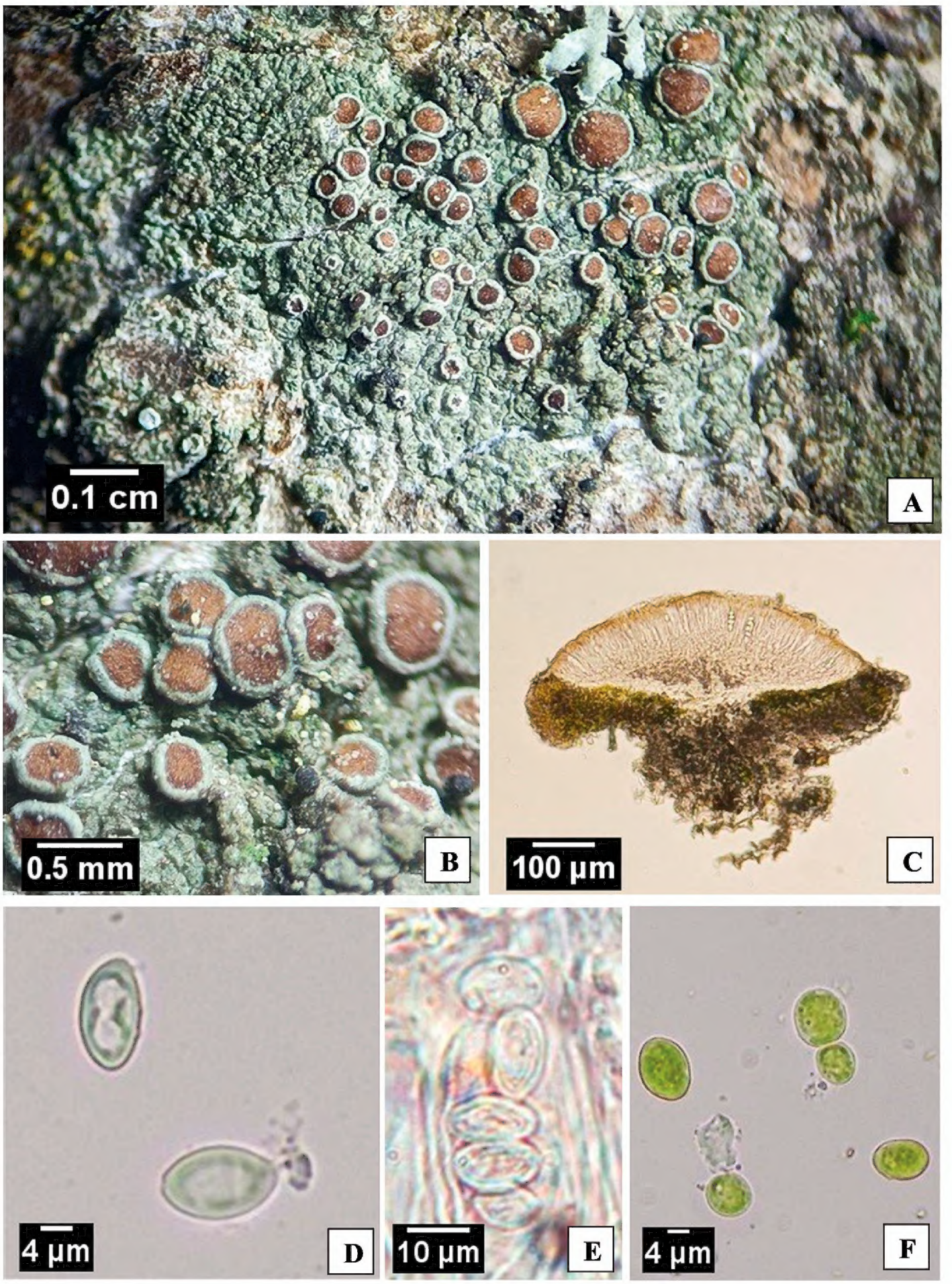

Figure 5. *Lecanora chlarotera* **A** thallus **B** apothecia **C** cross-section of apothecium **D** ascospores in water **E** asci containing ascospores **F** chlorococcoid photobionts.

L. campestris (Schaer.) Hue, *L. allophana* (Ach.) Nyl., and *L. glabrata* (Ach.) Nyl., forming a clade with high bootstrap support (82% ML).

Notes. *Lecanora chlarotera* is a common epiphytic species within the *L. subfusca* complex, widespread in Europe and North America, growing on nutrient-rich bark

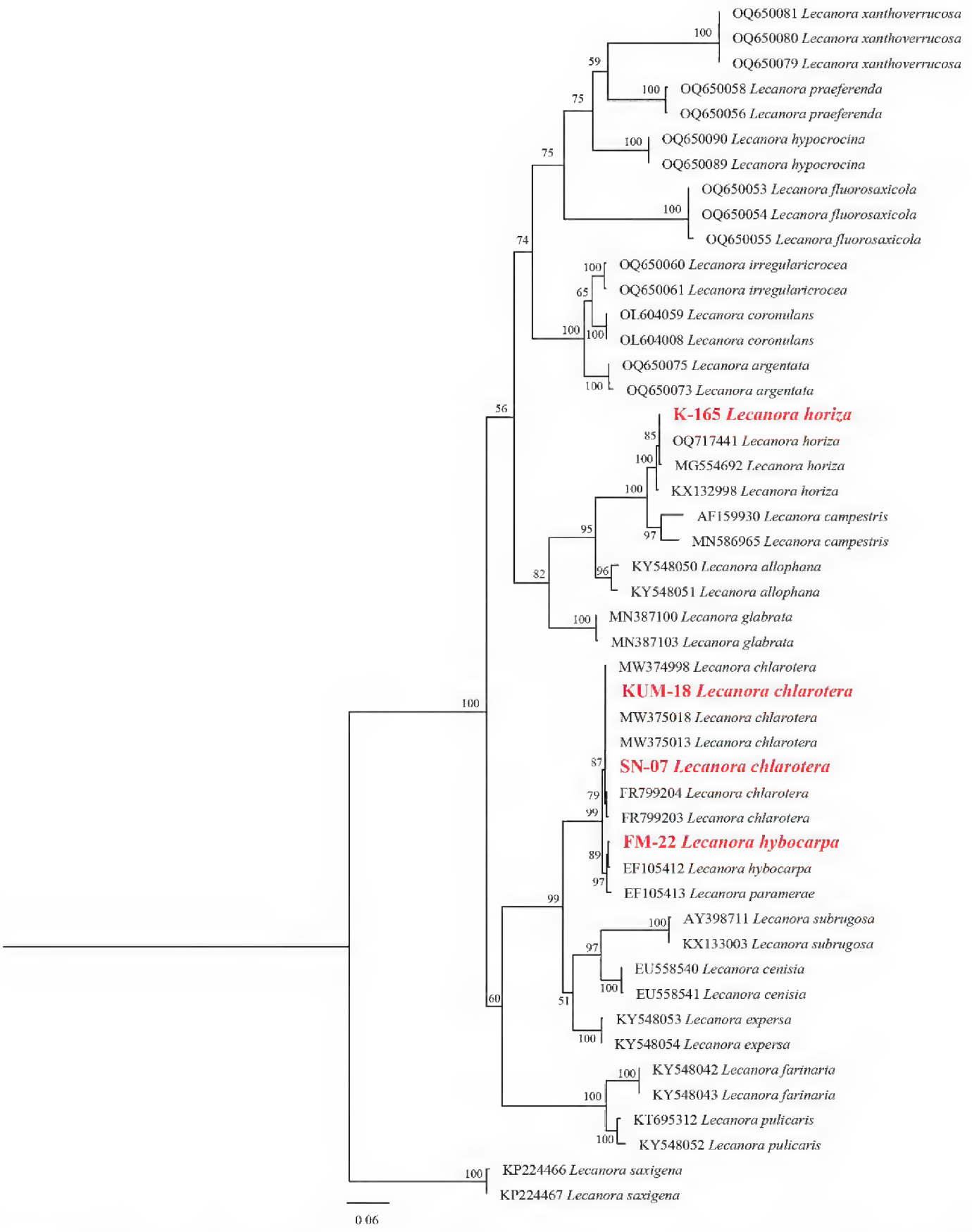

Figure 6. Molecular phylogenetic analysis of *Lecanora* spp. by maximum likelihood (ML) method based on nrITS sequences. Bootstrap values >50% based on 1000 replicates for ML are shown above the branches and novel sequences generated during this study are in bold.

from lowlands to mountainous regions (Bolognini 1992). The morpho-anatomical characteristics of the Pakistani specimen agree with the published description of *L. chlarotera* from the Czech Republic, except slightly smaller apothecia 0.3–0.8 mm (vs. 0.5–1 mm) and smoother apothecial margins (vs. smooth to crenate) (Malicek 2014). *L. chlarotera* is both morphologically and phylogenetically related to *L. hybocarpa* (Tuck.) Brodo, but the latter one has larger apothecial discs and yellow-white

to grey thallus coloration (Cannon et al. 2022). Phylogenetically, *L. chlarotera* is also closely related to *L. paramerae* I. Martínez, Aragón & Lumbsch but differs in having smaller apothecia 0.3–0.8 mm (vs. 1–3 mm), pruinose apothecial disc (vs. epruinose) and taller hymenium 70–100 µm (vs. 60–70 µm) (Martínez et al. 1999).

Habitat and ecology. The sample (KUM-18) was found at an elevation of 2,500 m, growing on a bark in a cold, temperate mountainous region. The coldest month in this region is December, while June is the hottest month of the year, with average monthly temperatures of 3 °C and 25 °C, respectively, and rainfall varying between 1000 and 1200 mm. The second collection (SN-07) was made from Kaghan Valley which is drained by the River Kunhar originating from Lulusar Lake. The average rainfall for spring, summer and winter is 363, 655, and 190 mm, respectively. The maximum daily temperature of the region varies from 20 °C to 30 °C during the summer and averages 4 °C during the winter with moderate rainfall.

Distribution. This species is widespread in the Czech Republic, Europe, Japan and Turkey (Miyawaki 1988; Öztürk et al. 2010; Malicek 2014). From Pakistan, it is reported for the first time.

Materials examined. Pakistan • Khyber Pakhtunkhwa Province, District Upper Dir, Kumrat Valley, 35°33.6'N, 72°12.4'E; alt. 2,500 m a.s.l.; 23 Sep. 2023; M. Usman & A. N. Khalid leg.; on bark; KUM-18, ITS GenBank accession number PQ113695. Pakistan • Khyber Pakhtunkhwa Province, District Manshera, Kaghan Valley; 34°42.2'N, 73°26.2'E; alt. 2,454 m a.s.l.; 24 Aug. 2022; A. R. Niazi & M. Nadeem leg.; on bark; SN-07, ITS GenBank accession number PQ106505.

***Lecanora horiza* (Ach.) Nyl., Flora, Regensburg 66(7): 107 (1883)**
Fig. 11A

Notes. *Lecanora horiza*, another species within the *L. subfusca* complex, is commonly found on deciduous trees and shrubs and occasionally on conifers, growing in sunny, nutrient-enriched conditions (Bolognini 1992; Malicek 2014). It is distributed in California, Europe, and Mexico (Bolognini 1992). Previously, it was reported from Baragali, Dungagali, Kalabagh, Murree, and Swat (Aptroot and Iqbal 2012) and is new to Kohistan, Khyber Pakhtunkhwa, Pakistan.

Material examined. Pakistan • Khyber Pakhtunkhwa Province, Kohistan, Dassu; 35°35.0'N, 73°37.0'E; alt. 841 m a.s.l.; 12 Sep. 2020; K. Habib & A. N. Khalid leg.; on rock; K-165, ITS GenBank accession number PQ469044.

***Lecanora hybocarpa* (Tuck.) Brodo, Beih. Nova Hedwigia 79: 134 (1984)**
Fig. 11B

Notes. This species is characterized by a continuous to rimose-areolate thallus, large crystals in the thalline margin, pale brown to red-orange discs and granules in the epithecium that dissolve in K (Cannon et al. 2022). It is distributed across Europe, Spain,

and the USA (https://www.gbif.org/). In Pakistan, it was previously reported from Kalam, Swat (Aptroot and Iqbal 2012) and is now recorded from Diamer, Gilgit-Baltistan.

Material examined. Pakistan • Gilgit-Baltistan, District Diamer, Raikot; 35°23.9'N, 74°04.7'E; alt. 1200 m a.s.l.; 05 Aug. 2023; N. S. Afshan & A. R. Niazi leg.; on rock; FM-22, ITS GenBank accession number PQ469043.

***Peltigera polydactylon* subsp. *udeghe* Magain, Miądl. & Sérus. in Magain, Sérusiaux, Zhurbenko, Lutzoni & Miadlikowska, Herzogia 29(2/1): 518 (2016)**
Fig. 7

Description. Thallus foliose, lobate, rhizinate, up to 6 cm across, esorediate. Lobes: separate to imbricate, adnate, crisped and rounded at margin. Color: dark black to greenish when wet and dark brown when dry. Upper surface: smooth and shiny. Lower surface: pale brown with reticulate veins, rhizinate. Rhizines: abundant, dark brown to black, scattered, fasciculate or bushy. Upper cortex: hyaline to brown, 40–60 µm thick. Algal layer: dark greyish to blackish, 100–120 µm thick, Photobiont *Nostoc*, 10–15 µm. Medulla: hyphae white, 250–300 µm thick, loosely interwoven. Lower cortex: hyaline to brown, 60–70 µm thick. Apothecia: rare, saddle-shaped, 6–7 mm in length. Disc: smooth, reddish to dark brown. Margin: crenulate to smooth, creamy to pale brown. Epihymenium: dark brown, 12–18 µm. Hymenium: light brown to hyaline, 90–130 µm. Hypothecium: creamy to light brown, 30–50 µm. Asci: hyaline, clavate, 8-spored, 70–105 × 9–14 µm. Ascospores: hyaline, filiform, rarely curved, 3–5 septa, 45–60 × 3–4 µm. Paraphyses: hyaline, simple, swollen with brown pigmented apex, 5–8 µm wide at apex.

Chemical study. K-ve, C-ve, KC-ve. Zeorin was detected through TLC.

Phylogenetic analysis. For phylogenetic analysis, sequences of the genus *Peltigera* were retrieved from GenBank based on their similarity to our sequences and those used in a study on the phylogeny of *Peltigera* by Juriado et al. (2017). A total of 35 ITS nrDNA sequences were analyzed during this study, including three newly generated sequences. The final aligned file was comprised of 709 nucleotides, among which 302 were conserved, 407 were variable, 406 were parsimony-informative, and one was singleton variant. *Solorina saccata* (L.) Ach. (MK503159 and MK503160) was chosen as an outgroup.

In our analysis, the newly generated sequence of *Peltigera polydactylon* subsp. *udeghe* (FM-15) is positioned within the polydactyloid clade of section *Polydactylon*. The Pakistani collection of *Peltigera polydactylon* (FM-15) clusters with sequences of the same species from China (MH976694 and OR178744) and the USA (KX365469). Similarly, the sequence of the Pakistani collection of *Peltigera praetextata* (KUM-10) groups with sequences from Norway (MH758516) and Russia (MH758518), forming a highly supported clade alongside *Peltigera canina* (L.) Willd. within section *Peltigera* (Fig. 8).

Notes. *Peltigera polydactylon* subsp. *udeghe* is characterized by non-tomentose upper surface, crisped, undulated, and usually phyllidiated thallus margins (Magain et al. 2016). It shares morphological and chemical similarities with *Peltigera polydactylon* subsp. *polydactylon* and *P. seneca* Magain, Miądl. & Sérus., but its thallus consistently exhibits phyllidia at the margins of lobes (Magain et al. 2016). From Pakistan, it is

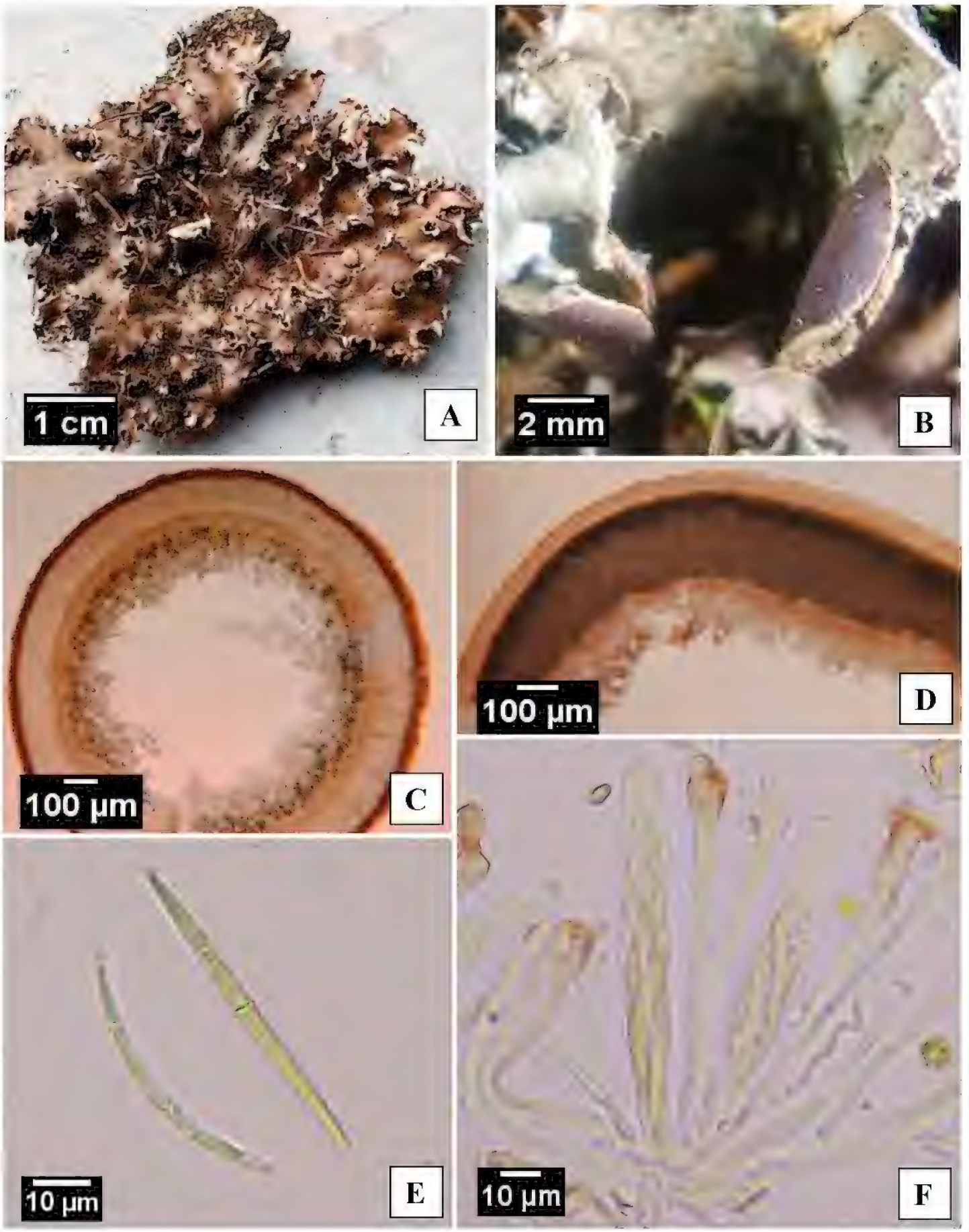

Figure 7. *Peltigera polydactylon* **A** thallus **B** apothecia **C** cross-section of apothecia **D** cross-section of thallus **E** ascospores **F** asci containing ascospores.

previously reported as *Peltigera polydactyla* (Neck). Hoffm. from Sharan (Kaghan) and Bahrain (Swat) (Aptroot and Iqbal 2012).

Habitat and ecology. Found at a high altitude on damp and detritus soil, not completely exposed to sunlight. This area, dominated by a mix of coniferous and deciduous forest trees, exhibits annual precipitation of 437 mm, and the temperature is 16 °C.

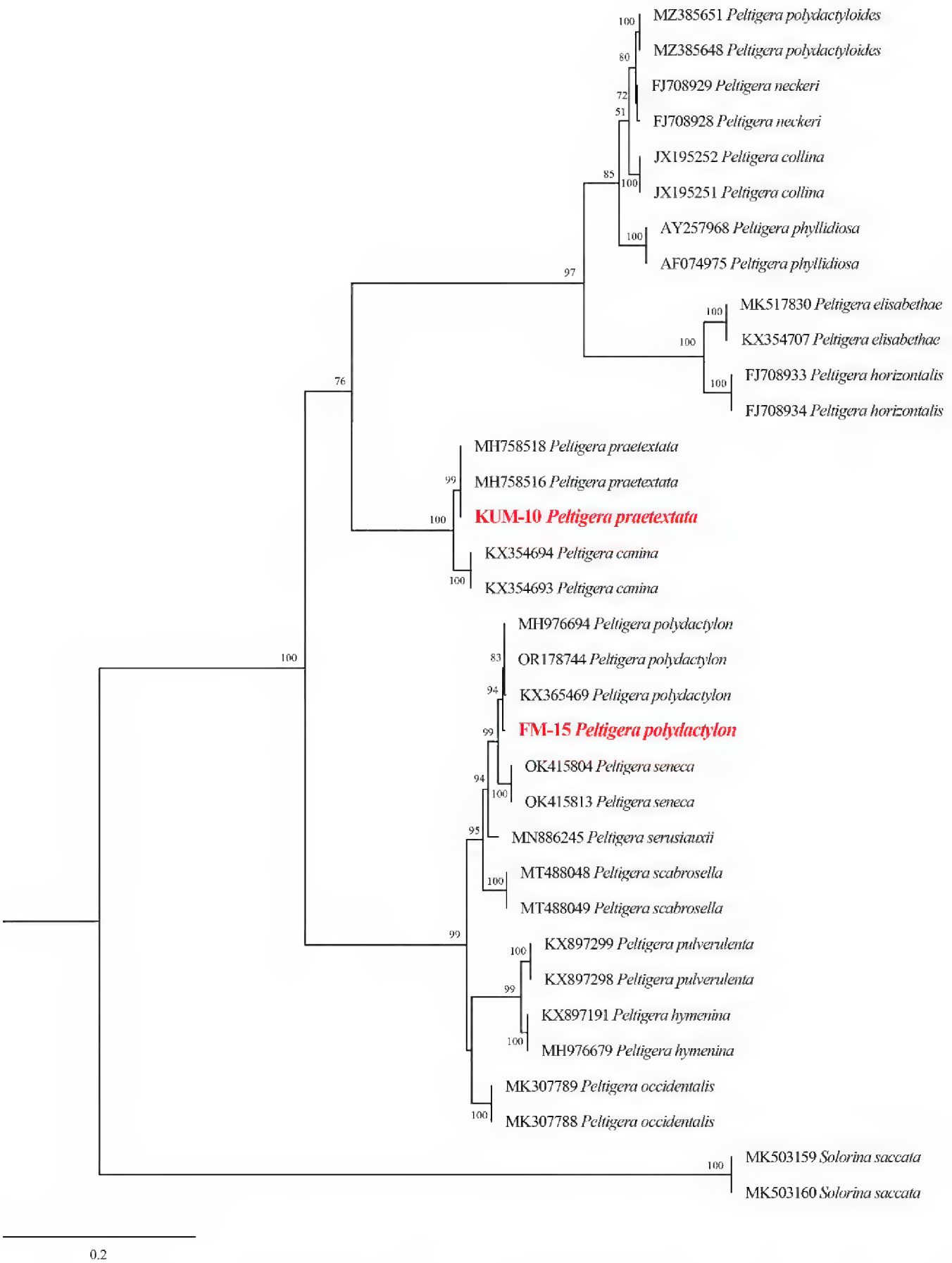

Figure 8. Molecular phylogenetic analysis of *Peltigera* spp. by maximum likelihood (ML) method based on nrITS sequences. Bootstrap values >50% based on 1000 replicates for ML are shown above the branches and novel sequences generated during this study are in bold.

Distribution. This species is widespread in Australia, Canada, New Zealand, the U.S.A and eastern Northern Asia (Magain et al. 2016).

Material examined. Pakistan • Gilgit-Baltistan, District Diamer, Fairy Meadows; 35°23'12.67"N, 74°35'02.98"E; alt. 3,300 m a.s.l.; 05 Aug. 2023; N. S. Afshan & A. R. Niazi leg.; on soil; FM-15, ITS GenBank accession number PQ469041.

***Peltigera praetextata* (Flörke ex Sommerf.) Zopf, Ann. Chemie 364: 299 (1909)**
Fig. 11C

Notes. This species is characterized by tomentose lobe margins along with laminal and marginal phyllidia (Goward 1995). It is morphologically and phylogenetically similar to *P. canina* (L.) Willd., but the latter differs in having branched rhizines and lacking isidia (Wei et al. 2009). Its distribution spans China, Europe, India, Japan, Nepal, New Zealand, North America, South Korea, and the United States (Awasthi and Joshi 1982; Park 1990; Wei 1991; Vitikainen 1994; Galloway 2000; Brodo et al. 2001; Hur et al. 2005). In Pakistan, previous collections were made from Utror, Swat (Aptroot and Iqbal 2010), while the new specimen was collected from Kumrat Valley. This study provides the first molecular confirmation of *Peltigera praetextata* from Pakistan.

Material examined. Pakistan • Khyber Pakhtunkhwa Province, District Upper Dir, Kumrat Valley, 35°33.6'N, 72°12.4'E; alt. 2,500 m a.s.l.; 23 Sep. 2023; M. Usman & A. N. Khalid leg.; on soil; KUM-10, ITS GenBank accession number PQ469042.

***Usnea lapponica* Vain., Meddn Soc. Fauna Flora Fenn. 48: 173 (1925) [1924]**
Fig. 9

Description. Thallus fruticose, pendent, tufted, erect, attached by a basal holdfast, up to 6 cm long. Branches: irregular, black at tips, segments terete, anisotomic dichotomous, longitudinally cylindrical, thin toward apex, up to 1 mm in diameter. Papillae: present on main branches, unevenly distributed, frequent. Color: thallus pale yellowish green to pale grey-green when dry, no change when wet. Cortex: prosoplectenchymatous, inner layer hyaline, outer greyish brown, 10–25 µm thick. Photobiont: 8–14 µm in diameter, globose to sub-globose. Medulla: loosely arranged, hyphae white. Soralia: superficial, discrete, excavated, mostly on thick branches, rounded to slightly irregular, soredia farinose and white.

Chemical study. K+ve (yellow), C-ve, KC+ve (yellow). Usnic acid and salazinic acid were detected through TLC.

Phylogenetic analysis. For phylogenetic analysis, sequences of the genus *Usnea* were retrieved from GenBank based on their similarity to our sequences. A total of 31 ITS nrDNA sequences were analyzed during this study. The final aligned file was comprised of 503 nucleotides, among which 397 were conserved, 104 were variable, 88 were parsimony-informative, and 16 were singleton variants. *Protousnea magellanica* (Mont.) Krog (DQ985192 and KR995309) was chosen as an outgroup. In the ITS phylogram, the sequence of the Pakistani *Usnea lapponica* specimen forms a clade along with sequences of the same taxon reported from Austria (KJ406267), India (KJ406266), Sweden (KJ406265), and Switzerland (KX132930) (Fig. 10).

Notes. *Usnea lapponica* is characterized by an erect to pendent fruticose thallus with deeply excavated soralia and farinose soredia (Halonen et al. 1998). For morphological comparison, the characteristics of the Pakistani sample were compared with the description of *U. lapponica* given by Halonen et al. (1999), which shows that both of

Figure 9. *Usnea lapponica* **A, B** fruticose thallus **C** branches of the thallus **D** cross-section of the branch showing central strand.

them share the same characteristics such as a highly branched thallus, slender branches, a thinner cortex, and a thicker central axis but differ from each other due to having different branching patterns (anisotomic-dichotomous vs. isotomic-dichotomous) (Halonen et al. 1999).

Habitat and ecology. Corticolous, found at an elevation above 3,228 m, growing in a cold and dry temperate mountainous region, with a mean maximum and minimum temperature of 22 °C and -18 °C, respectively, and an annual rainfall varying between 254 and 400 mm. The dominant vegetation includes *Picea smithiana* (Wall.) Boiss, *Pinus wallichiana* A.B.Jacks., and *Betula utilis* D.Don.

Distribution. This species is widespread in Europe, Finland, Iran, North America and Russia (Halonen et al. 1999; Seaward et al. 2004). From Pakistan, it is reported for the first time.

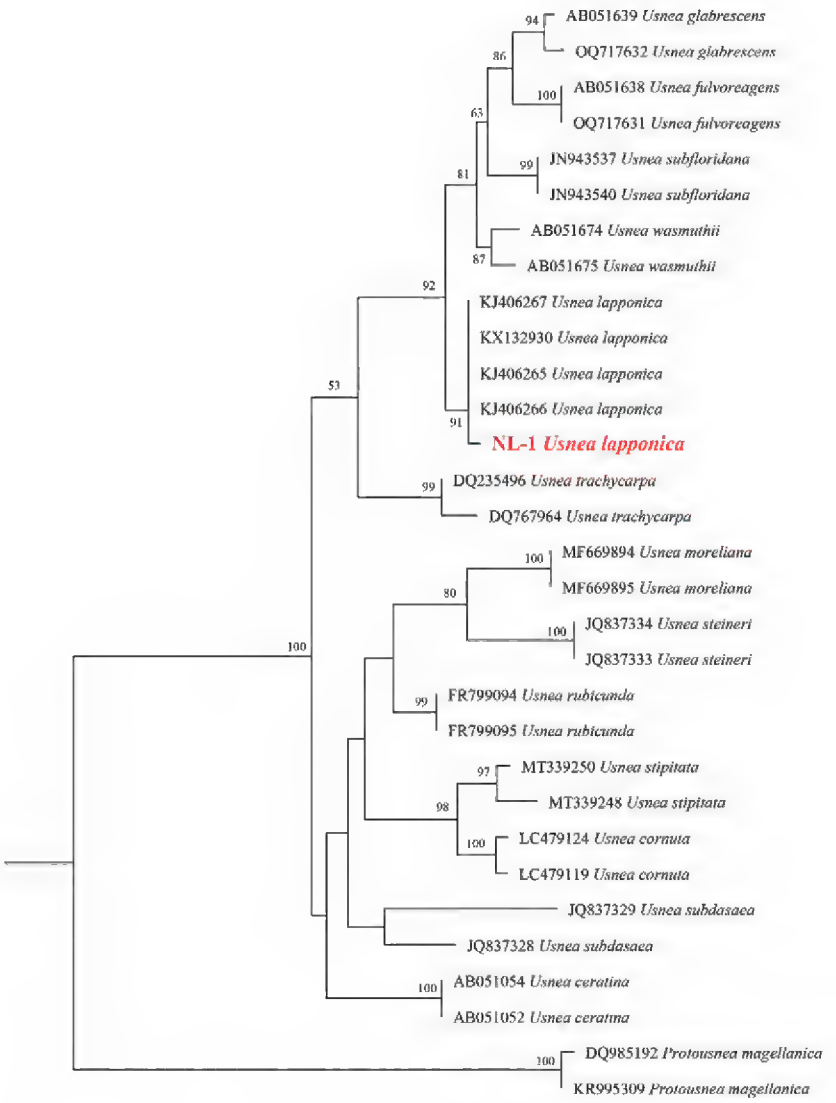

Figure 10. Molecular phylogenetic analysis of *Usnea* spp. by maximum likelihood (ML) method based on nrITS sequences. Bootstrap values >50% based on 1000 replicates for ML are shown above the branches and novel sequence generated during this study is in bold.

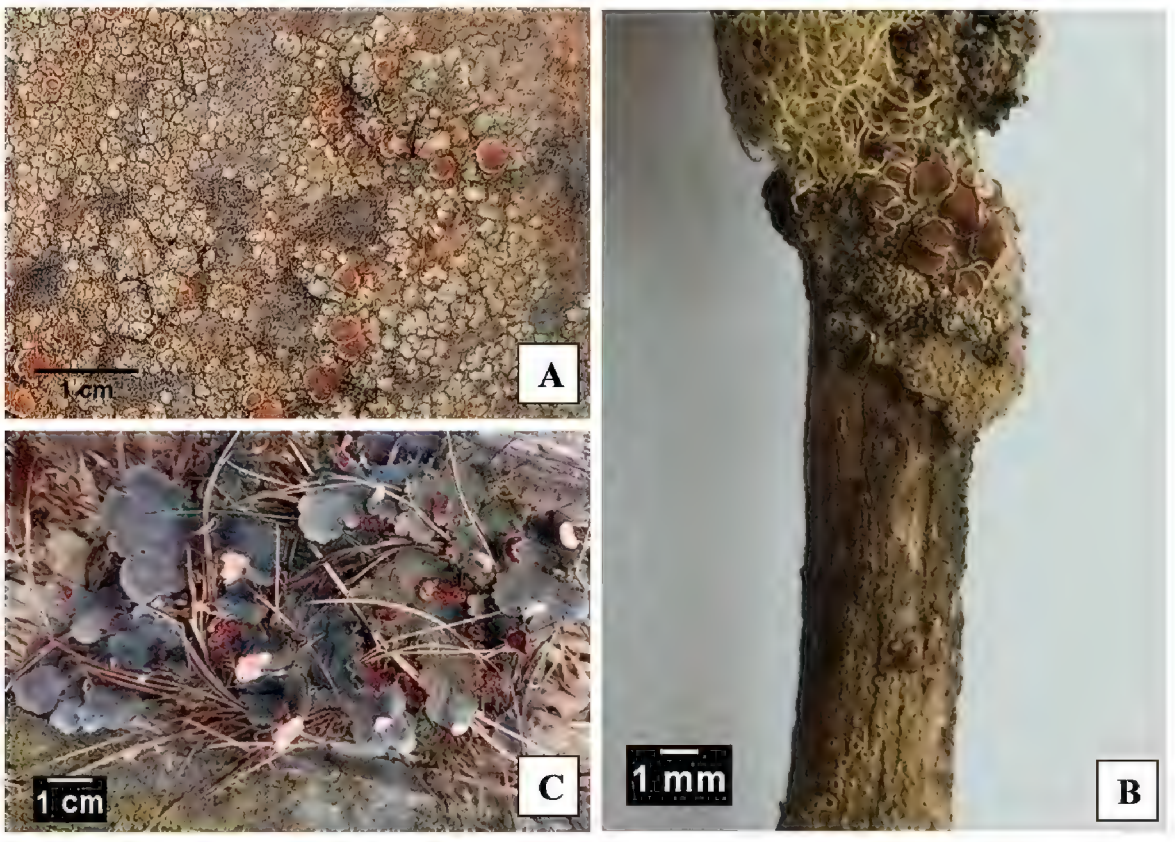

Figure 11. Thalli of selected lichen species showing distinct morphological characteristics: **A** *Lecanora horiza* **B** *Lecanora hybocarpa* **C** *Peltigera praetextata*.

Material examined. Pakistan • Gilgit-Baltistan, District Gilgit, Naltar Valley, Blue Lake; 36°14.2'N, 74°06.1'E; alt. 3,228 m a.s.l.; 30 Aug. 2023; U. F. Ahmad, M. Usman & A. N. Khalid leg.; growing on bark; NL-1, ITS GenBank accession number PQ113701.

Conclusion

In this study, we described several lichen species from Pakistan, specifically from Khyber Pakhtunkhwa and Gilgit-Baltistan. These lichen samples were analyzed using morpho-anatomical, molecular, and biochemical methods, focusing on adding to the lichen flora of the country and gaining a more comprehensive understanding of their distribution patterns and ecology relative to global lichen flora. The findings not only contribute to the knowledge of lichen biodiversity in Pakistan but also highlight the need for further extensive surveys to reveal the full extent of the country's lichenized fungi.

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